Green defense against thrips: Exploring natural products for early management of western flower thrips

Mouden, S.

CHAPTER SIX

WATER DIPPING OF INDOLE-3-BUTYRIC ACID COATED CHRYSANTHEMUM CUTTINGS CONFERS PROTECTION TO INSECT HERBIVORES

Abstract

Sanae Mouden[1], Kirsten A. Leiss[2], Henriette Uthe[3,4] and Peter G.L. Klinkhamer[1]

[1] Plant Sciences and Natural Products, Institute of biology, Leiden University, P.O. Box 9505, 2300 RA, the Netherlands.
[2] Wageningen University & Research, Business Unit Horticulture, Violierenweg 1, 2665 MV Bleiswijk, the Netherlands.
[3] Molecular Interaction Ecology, German Center for Integrative Biodiversity Research (iDiv), Halle-Gena-Leipzig, Deutscher Platz 5e, 04103 Leipzig, Germany.
[4] Friedrich Schiller University Jena, Institute of Biodiversity, Dornburger-Str. 159, 07743 Jena, Germany.

Chrysanthemum is a major ornamental plant species. Among the most important production constraints are biotic stresses, in particular thrips and leaf miner infestations form a prominent hazard during its vegetative state. Auxins are commonly used for commercial propagation of chrysanthemums by stem cuttings. However, recent studies suggest that these root-promoting hormones may also affect plant defense responses. The underlying motive of this study stems from the serendipitous observation that water dipping of auxin-coated cuttings beneficially affected thrips herbivory. Therefore, the primary objective of this investigation was to explore the role of indole-3-butyric acid (IBA) in relation to western flower thrips susceptibility in chrysanthemum. We observed contrasting findings concerning the physical presence of IBA and its' role in promoting susceptibility of cuttings to thrips. Nonetheless, we repeatedly demonstrated considerable protection, in some experiments up to 37%, against thrips as well as leaf miner upon water dipping of IBA-coated cuttings. Assessment of polyphenol oxidase activity (PPO), 14 days after dipping treatment, suggests that neither direct induction nor priming of plant defenses are involved. Overall, however, results were highly variable and thus, do not allow us to deduce conclusive statements concerning the involvement of auxin-mediated defense responses. An explanation for the variation may be the large phenotypical variation of cuttings generated from mother stock plants. Future experiments aiming at understanding the early signaling events, including hormonal signaling networks, from a more holistic perspective, may help to explain the physiological basis involved in conferring protection against herbivores.

Keywords: auxin, western flower thrips, *Liriomyza trifolii*, resistance, polyphenol oxidase

Introduction

Chrysanthemum morifolium (Ramat) is a semi hardy herbaceous, perennial flowering plant and belongs to the family of Asteraceae (formerly known as Compositeae). Chrysanthemums are among the most important commercially grown greenhouse ornamentals worldwide and are extensively used as cut flowers and as pot plants (Fletcher, 1992; Machin, 1996; Xia et al., 2006). Classical breeding programs have mainly focused on improving various characteristics to enhance ornamental values, including flower colour, size and shape. Driven by such consumer needs, breeders are often under pressure to supply novel varieties within a restricted timeframe and with very specific choices, leaving few options for altering other agronomic traits. In addition to the limited gene pool, chrysanthemums are hexaploids and genetically highly heterozygous, thus complicating the development of resistant varieties and plant novelties at the same time (Teixeira da Silva et al., 2013). Consequently, many commercial varieties often lack resistance traits to biotic and abiotic stresses.

Cultivars grown under greenhouse conditions are constantly challenged by a number of arthropod infestations among which susceptibility to the western flower thrips (WFT), *Frankliniella occidentalis* (Pergande), is one of the main constraints in the year round production of quality chrysanthemums (Guldemond et al., 1994; Mouden et al., 2017a). Thrips feed by piercing plant tissues with their needle-shaped mouthparts, causing two types of feeding damage (de Jager et al., 1993). Growth damage is caused by feeding on actively growing tissue, whereas feeding on older, expanded tissue causes cells to become filled with air, which imparts a silvery appearance. The latter, known as silver damage, significantly affects product appearance and, hence reduces marketability. Insecticides have been a predominant management strategy, especially for high-value aesthetic ornamentals like chrysanthemum that require nearly zero level damage thresholds. However, once established in a greenhouse, control of thrips may be difficult to obtain due to their thigmotactic behavior and the limited number of active substances available (Mouden et al., 2017a). Management of pests early in the production cycle is, therefore, extremely important to prevent populations from building up to economically damaging levels.

A second important pest species in ornamental greenhouses is *Liriomyza trifolii* (Burgess) (van Dijk et al., 1992). Adult flies of the American serpentine leaf miner puncture leaves for feeding and oviposition. Feeding of larvae on the mesophyll leads to the formation of serpentine mines. When mature, they exit the leaf to pupate in the soil. (de Jong and van de Vrie, 1987). Both larval and adult stages can cause a decrease in the photosynthetic area, facilitate entry of plant pathogens, and adversely affect product appearance and yield.

Cultivars of commercially grown chrysanthemum are predominantly asexually propagated through vegetative terminal cuttings in order to generate genetically identical clones of the mother stock plants. Successful clonal propagation starts with adventitious root (AR) formation in stem cuttings and involves a large set of endogenous and exogenous factors (Druege et al., 2016; Druege et al., 2019). Auxins, a class of plant growth regulating hormones, affect many crucial plant physiological processes and are key determinants of rooting propensity of cuttings (Bellini et al., 2014; Pacurar et

al.,2014). Excision-induced formation of ARs is generally marked by accumulation of the endogenous auxin indole-3-acetic acid (IAA). Although IAA is able to stimulate ARs, indole-3-butyric acid (IBA) promotes rooting more efficiently, a feature partially due to its greater stability to light compared to IAA. Therefore, external application of IBA as talc or water diluted formulations is preferred in clonal propagation of commercial species.

In search of effective dipping treatments to protect cuttings during their vegetative stage, we serendipitously observed a reduction in thrips-associated feeding damage following a dipping treatment of the auxin-coated cuttings in water. Indeed, in spite of the fact that the phytohormone auxin has classically been implicated in developmental processes, recent studies demonstrate that auxin also affects a multitude of plant defense responses through complex interactions among multiple hormone pathways (Kazan and Manners, 2009; Erb et al., 2012; da Costa et al., 2013). Extensive cross talk between auxin and JA exists in both directions as these two hormones share many commonalities in their molecular mechanisms for hormone perception and signal transduction (Pérez and Goossens, 2013). However, only a few reports are available regarding the exogenous effects of auxin on JA synthesis, which in addition, appear to be inconclusive. Auxin acts either synergistically or antagonistically with other hormones to trigger cascades of events leading to AR initiation and development (Lakehal and Bellini, 2019). Several lines of evidence indicate that auxins antagonize JA biosynthesis and signaling. External application of auxin suppressed herbivory-induced accumulation of jasmonates (Baldwin et al., 1997). Likewise, a number of wound induced responses, including the expression of jasmonate-dependent proteinase inhibitor genes and vegetative storage proteins were negatively controlled by the level of auxin (Kernan and Thornburg 1989; DeWald et al., 1994). Down regulation of JA-responsive genes was also observed in auxin treated *Arabidopsis* plants (Rojo et al., 1998; Liu and Wang, 2006). Furthermore, auxin has been shown to induce expression of the JA signaling repressor JAZ1 (Grunewald et al., 2009). Although all these experiments suggest an inhibitory effect of auxin on JA biosynthesis, contradictory results have been reported recently with auxins inducing the expression of JA biosynthetic genes (Tiryaki and Staswick, 2002; Fattorroni et al., 2017). Auxin also mediated increases in JA concentration whereas, mutants with no functional auxin receptors showed a reduction in JA amounts, suggesting a synergistic function of auxin and JA (Grossman et al., 2004; Nagpal et al., 2005).

These results illustrate the complexity of auxin-induced changes and strongly suggest their importance in plant stress responses, particularly toward biotic stresses. Numerous studies, mainly from the analysis of mutant phenotypes, have indeed demonstrated the involvement of auxins in disease development (Kazan and Manners, 2009; Robert-Seilaniantz et al., 2011; Čarná et al., 2014). The changes incurred in plants as a result of these plant growth regulators may, therefore, likely affect plant insect-relationships. However, these effects have been poorly explored (Erb et al., 2012). Given their importance in commercial plant propagation, the impact of auxin on insect herbivory deserves more attention if we are to understand their role as defense regulators. In the present study, we investigated host plant resistance against WFT in auxin-coated chrysanthemum cuttings. In this context, we hypothesized that chrysanthemum susceptibility against thrips herbivory could be

attributed to attenuation of induced defenses by exogenous auxins, possibly through its antagonistic effect on JA. Alternatively, the observed reduction of silver damage symptoms in chrysanthemum cuttings may equally well result from a synergistic mode of action of auxin with JA by improving the uptake of externally applied auxin upon water dipping. Therefore, we aimed to investigate the effect of IBA and water dipping primarily in relation to thrips resistance. Moreover, we explored plant defense responses triggered upon treatments by measuring the expression of the defense-related marker protein polyphenol-oxidase (PPO) and several defense- and growth- related phytohormones.

Materials and Methods

Plant material and growth conditions

Chrysanthemum plants (*Chrysanthemum morifolium* Ramat) of the cultivar Baltica, which is susceptible to WFT, were used in all experiments and were kindly provided by Deliflor Chrysanten B.V. (Maasdijk, the Netherlands). Unrooted cold-stored chrysanthemum cuttings were imported from different production facilities located in Ethiopia (E) or Uganda (U). As a standard procedure for the production of commercial cuttings, the basal cut ends of freshly pinched cuttings were pre-coated with rooting powder by greenhouse workers, in order to stimulate root growth. Rooting hormone powder consisted of 0.4% indole-3 butyric acid in talc (Chryzoteck beige 0.4%) and was applied as a dry powder formulation. Unrooted chrysanthemum cuttings were approximately 5 cm long with three to four nodes. Commercial pre-coated cuttings were individually planted in plastic trays (4×4×6 cm) containing a 3:1 mixture of potting soil (Horticoop, Lentse Potgrond, the Netherlands) and vermiculite, pre-moistened to saturation using tap water. During the initiation phase of rooting, cuttings were covered with a transparent polyethylene plastic bag fitted over a plastic sliding tray (60×40×55 cm; Beekenkamp Verpakkingen B.V., Maasdijk, the Netherlands) to maintain humidity and prevent desiccation of cuttings. After a rooting period of 11 days, the cover was removed and cuttings were transplanted to plastic pots (Ø 11 cm; Pöppelmann, Germany) containing potting soil, 10 g/L vermiculite and 1.5 g/L osmocote slow release fertilizer (Scott, Scotts Miracle-Gro, Marysville, Ohio, USA: 15:9:11 NPK). Cuttings were grown in a climate room provided with 113.6 µmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR) and a light/dark cycle of 16/8 h at 20°C and 70% relative humidity (RH) in a completely randomized design. Watering was applied every two days. At 14 days after planting, plants were randomly subjected to a non-choice whole plant thrips bio-bioassay or sampled for chemical analyses as described below.

Non-choice whole plant thrips bioassay

A non-choice whole plant bioassay was used to evaluate resistance against WFT (Leiss et al., 2009). Two-week old cuttings were individually placed in thrips-proof cages consisting of a plexiglass cylinder (50 cm height, 20 cm diameter) closed at one end with a displaceable ring of nylon gauze of 120 µm mesh size. Plants were randomly placed in a climate-controlled growth chamber at a constant temperature of 25 °C, a photoperiod of 16L:8D and 70% RH. For infestation, adult thrips were collected

in glass jars using a mouth-operated aspirator. Western flower thrips were obtained from a continuous mass-rearing on flowering plants of the susceptible chrysanthemum variety Euro Sunny and were maintained in a climate room at 25 °C and 70% RH. Twenty adult thrips, consisting of eighteen females and two males, were released inside the cage, simulating high-density infestations. Seven or 14 days after infestation, cuttings were visually inspected for thrips feeding damage, hereafter referred to as 'silver damage'. Silver damage, expressed as damaged leaf area in mm^2, was evaluated in all leaves. Whole plant cumulative silver damage of plants is presented in the graphs.

Non-choice whole plant leaf miner bioassay

Leaf miners, *Liriomyza trifolii* (Burgess), were obtained from a continuous mass rearing on a susceptible cultivar of chrysanthemum (Ultra Light) in a climate room provided with L16:D8 and 60% RH at 23 °C. In order to obtain pupae, infested plants containing third instar larvae were placed horizontally. One-day old flies were shortly anesthetized by CO_2 and sorted by sex using a fine sable paintbrush prior to release onto the plants. Two unmated males and females each, were placed into a small cage with chrysanthemum cuttings. The adult leaf miners were allowed to deposit eggs on the plants for 24 hours, after which they were removed from the cage. All plants, free of adult leaf miners, were then moved to another clean climate room (L16:D8, 70% RH, at 25 °C) and placed randomly. The total number of mines on the plants were counted 3 days after leaf miner release to prevent overlap in formation of mines and thus, allow for more accurate counts of individual mines. Plants were harvested by cutting them at the crown level and transferring them into individual Ziploc bags. Subsequently, these bags were placed in a climate chamber at 20 °C for pupae to develop. The number of pupae was scored one week after harvesting using a dissecting microscope (25x). Plants were carefully checked for remaining pupae in leaf tissue, which were indicated as non-emerged pupae.

Dipping treatments

Bio-insecticidal dipping of unrooted cuttings

The use of natural compounds with low risk profiles offer a simple and a cost-effective opportunity for sustainable pest management. Among them, β-alanine has been reported confer resistance against WFT (Leiss et al., 2013). Therefore, bio-insecticidal dips were evaluated as a pre-treatment of unrooted chrysanthemum cuttings to enhance resistance against thrips. Treatments were based on the results of preliminary trials in the laboratory. The basal cut end (~1 cm) of unrooted cuttings were individually dipped in an aqueous solution of beta-alanine at a final concentration of 100 mg/ml. Water dipping treatment was considered as control whereas, a non-dipped group of unrooted cuttings was included as a negative control group. Furthermore, the effect of exogenously applied jasmonic acid was included as a positive control. Based on preliminary experimental results, a dipping time of 60 minutes was selected as an effective duration for JA treatment. The JA stock solution (1.19 M) was prepared by dissolving 2.5 mg in 1 ml absolute ethanol which, prior to use, was

diluted to a final working concentration of 5 mM. Following dipping treatments, cuttings were planted in a mixture of pre-moistened soil and vermiculite. Two weeks after rooting, chrysanthemum plants were subjected to a non-choice whole plant bio-assay as previously described. Various dipping durations were evaluated (e.g. 30, 45 and 60 min) but pooled silver damage data were used for analysis.

Water dipping of IBA pre-coated cuttings in relation to thrips resistance

To investigate the role of auxins in chrysanthemum susceptibility and to explore the potential of water dipping treatments for resistance improvement, commercial cuttings pre-coated with IBA (Chryzoteck beige 0.4%) and non-coated control cuttings free of IBA were used for this experiment. The basal end (~1 cm) of the cuttings were individually dipped in water for 30, 45 or 60 minutes, whereas non-dipped cuttings served as negative control (indicated as t=0). The cuttings were immediately planted and randomly grown under conditions described above. Two weeks after rooting, chrysanthemum plants were subjected to a non-choice whole plant bio-assay or sampled for dry weight measurements, with ten replications per treatment.

Water dipping of IBA pre-coated cuttings in relation to leaf miner resistance

Furthermore, we evaluated the involvement of auxins in relation to leaf miner resistance. To examine the role of IBA in leaf miner susceptibility, unrooted Baltica cuttings with and without IBA coating were grown for 2 weeks after which they were infested with four adult leaf miners for a period of 24 hours (n=15). In a second experiment, the effect of water dipping was investigated in the presence of IBA coating. Commercial cuttings pre-coated with IBA (Chryzoteck beige 0.4%) were dipped in water for various durations (30, 45, 60 minutes), whereas non-dipped cuttings served as negative control (indicated as t=0). Additionally, a positive control was included by dipping the basal cut ends for 60 minutes in a solution of 5 mM jasmonic acid. After treatment, all cuttings were directly planted in soil. After the rooting period, cuttings were subjected to a leaf miner bioassay (n=13) or sampled for polyphenol oxidase measurements as described elsewhere (n=8 to 10). After scoring pupae emergence the plants were transferred to paper bags and dried in an oven at 50 °C for at least 3 days for measurements of plant dry mass .

Standardization of IBA applied powder formulation

The objective of the present experiment was to standardize hormone powder application in order to potentially reduce IBA-induced response variations among cuttings. For powder formulations the main variables were those factors determining the amount of powder adhering to the cutting base and epidermis, including pre-dipping in water, retaining the powder through careful handling and standardizing the diameter of the cutting insertion (5 mm) at the soil surface to prevent loss of hormones. Rooted Baltica cuttings, planted in small compost blocks of 64 cm^3, were manually pinched to obtain stem cuttings of 5 cm in length. The full factorial experiment contained two factors, each with four levels with main treatments being auxin application (no dip, 0.4 and 0.8% IBA and

talc) and water dipping time (0, 30, 45 and 60 minutes). Indole-3-butyric acid was applied as a dustable talc-based powder formulation to basal cut ends. Talc powder, primarily consisted of magnesium silicate ($Mg_3H_2(SiO_3)_4$, Sigma-Aldrich with particle diameters measuring between 1.9 and 2.3 µm) and was used as an inert carrier for dry powder formulations and was homogeneously mixed with IBA at a final concentration of 0.4 and 0.8% (w/w). To satisfactorily adhere to the cut ends and to provide a medium of contact through which the hormone could enter plant tissues, the basal cut ends were pre-wetted with water. Subsequently, cut bases were dipped in 0.4 % IBA, 0.8% IBA or talcum powder. In order to ensure precise application the amount of powder that was applied to the cut base of the stem was standardized to approximately 150 mg. Excess powder was brushed off. Other sets of cuttings were untreated and served as negative control. To mimic the proposed commercial application and to keep cuttings turgid, they were stored in sealed plastic bags under cold (4 °C) and dark conditions for a week prior to water dipping treatments. For water dipping treatments, unrooted cuttings were vertically placed in glass vials filled with a layer of water at room temperature, covering 1 cm of their basal cut end. Cuttings were individually dipped in water for 30, 45 or 60 minutes, whereas control cuttings were not dipped (t = 0 min). All cuttings were planted on the same day, after their respective basal water dip treatments, in a 3:1 potting mix consisting of potting soil with vermiculite. Fifteen cuttings were treated as a sampling group of which ten cuttings from each treatment group were subjected to a non-choice whole-plant thrips bioassay and five cuttings per treatment were used for plant hormone analysis as described below. For statistical analysis, data for silver damage, hormone concentrations and dry mass were pooled (30-45 and 60 min) for each coating treatment

Comparing dry-dip rooting powder with water-based rooting solution

The efficacy of two different application methods for IBA was investigated to avoid potential confounding effects exerted by talc-powder. The basal cut end of commercially provided unrooted Baltica cuttings, pre-coated with IBA, was dipped in water for 30 minutes. The commercial rooting powder Chryzotek beige 0.4% contains 4000 parts of indole-3-butyric acid to a million parts of talc and thus, basal liquid dips were performed using concentrations equimolar to powder applied IBA. To this end, eight water soluble IBA tablets of 50 mg each (Rhizopon AA) were dissolved in 100 ml MilliQ water at RT to obtain a final concentration of 4000 ppm (Rhizopon, Hazerswoude-Rijndijk, the Netherlands). Cuttings, free of hormone powder were inserted in a floating mat of which approximate 1 cm of the basal cut end was dipped in the IBA suspension for 30 minutes. Water soluble IBA solution was constantly stirred to avoid precipitation. After dipping, cuttings were grown as previously described. Each treatment consisted of 15 replicates. Two weeks after rooting cuttings were subjected to a non-choice whole-plant thrips bioassay .

Effect of dipping on different forms of induced resistance in chrysanthemum

To evaluate whether reductions in thrips associated feeding damage operate in a JA-dependent manner, polyphenol oxidase activities were measured. Forty IBA-coated cuttings were dipped in

water for 30 minutes. Control cuttings, pre-coated with IBA, were directly inserted in a pre-mixture of soil and vermiculite (n=40). Two weeks post treatment cuttings were randomly divided in two groups of 20 each and were either infested with thrips or sampled for PPO measurements (i.e. direct induction). To demonstrate whether the underlying mechanisms govern direct induction of defenses or prime for a potentiated response, cuttings were also sampled for PPO assessment after thrips infestation.

In parallel to this experiment, an additional 280 cuttings were subjected to the same treatments as above to determine whether defense responses were time-dependent. For this time course experiment, chrysanthemum cuttings pre-coated with IBA were subjected to control (no dipping) or water dipping (30 min) treatment. At 14 days after the start of the dipping treatments, all leaves were sprayed with 2 mL of 7.5 mM MeJA (Sigma-Aldrich) or treated with the corresponding mock solution consisting of 0.8% aqueous ethanol. JA-associated defenses were artificially induced using methyl jasmonate (MeJA) because inductive effects on PPO bioactivities were observed to be more consistent than herbivore induction. Ten cuttings from a given treatment were periodically sampled for PPO measurement at 0 (i.e. before hormone treatment), 12, 24, 36, 48, 72 and 168 hours (7 days) after hormone application.

Polyphenol oxidase (PPO) activity

Polyphenol oxidase (PPO) activity was measured following the method of Stout et al. (1998) with slight modifications. Two weeks after treatment (i.e. before infestation) or one week post infestation, the third leaf from the bottom was sampled. Fresh leaf material was ground using a tissue lyser (Qiagen, Hilden, Germany) and stored at –80 °C until analysis for PPO activity. Hundred fifty milligrams of fine powder was extracted with 1.25 ml ice-cold potassium phosphate buffer (0.1 M, pH 6.8) containing 7% (w:v) polyvinyl polypyrolidine. To this homogenate, 0.4 ml of a 10% solution of Triton X-100 was added. Plant extracts were vortexed for 2 min and centrifuged at 11,000 g for 10 min at 4 °C. The resulting supernatant was used directly as an enzyme source using chlorogenic acid as a substrate. The reaction mixture consisted of 5 µl enzyme extract and 1 ml of 2.92 mM chlorogenic acid dissolved in 0.1 M potassium phosphate buffer at pH 8.0. The rate in change of absorbance of this mixture was spectrophotometrically measured at 470 nm for one minute (UV-1800 UV-VIS spectrophotometer, Shimadzu Europe GmbH, Duisburg, Germany). PPO activities were calculated from the linear slope and were reported as changes in absorbance values per min per gram of fresh weight.

Hormone analysis

To investigate the signaling pathways involved in auxin-mediated induction of defenses against WFT, we determined how coating and water dipping treatments influenced plant defense- and growth-related hormone levels. Two weeks after initial dipping treatment, prior to thrips infestation, the basal third leaf was sampled for hormone analysis (n=5). Analysis of jasmonic acid (JA), its biosynthetic precursor 12-oxo-phytodienoic acid (OPDA), jasmonic acid-isoleucine (JA-Ile), salicylic acid (SA),

abscisic acid (ABA) and auxin (indole-3-acetic acid, IAA) were performed following the procedures described by Machado et al. (2013) and Schäfer et al. (2016), with some modifications. Leaves were flash frozen in liquid nitrogen and stored at –80 °C until freeze-drying. Approximately 100 mg of frozen and homogenized leaf material was aliquoted in 2 ml Eppendorf tubes and extracted with 1 ml of methanol containing 40 ng of the phytohormone standards D_6-ABA (Olchemin), D_6-JA (HPC), D_6-JA-Ile (HPC), D_6-SA (Olchemin), and D_5-IAA (Olchemin). Samples were vortexed for 10 min and centrifuged at 14,000 rpm for 10 min at 4 °C. Subsequently, the supernatants were transferred to new Eppendorf tubes and evaporated to dryness in a vacuum-concentrator at room temperature. The residue was dissolved in 20 µl 70% aqueous methanol (v/v) for 5 min using an ultrasonic bath, and centrifuged 5 min at 14,000 rpm. The supernatants were transferred to glass vials and then analyzed by LC-MS/MS (EVOQ, Bruker Daltonics). One µl of each sample was injected onto a C_{18} Zorbax-Eclipse column (50 × 4.6 mm, 1.8 µm, Thermofisher). The mobile phase was comprised of LCMS-grade water (solvent A) and acetonitrile (solvent B), both containing 0.05% (v/v) formic acid. The program had a constant flow rate of 400 µl min^{-1} and consisted of 0–0.5 min 95% solvent A; 0.5–2.5 min 50% solvent A, and 50% solvent B; 2.5–3.5 100% solvent B; 3.5–4.5 min 95% solvent A. The column temperature was set at 42 °C. The cone, probe, and nebulizer gas were set at the following flow conditions (arbitrary units/temperature): 35/350 °C, 60/475 °C and 60 (arbitrary unit), respectively. The phytohormones were quantified using the signal of their corresponding internal standard and were expressed on the basis of fresh leaf weight.

Statistical analyses

Normal distributions were confirmed by Shapiro-Wilk tests and homogeneity of variances were determined by Levene's tests. Means were compared, where appropriate, using an unpaired Student's t-test or one-way ANOVA followed by Fisher's least significance difference (LSD) post hoc test. When assumptions were violated, data transformations were performed or differences in means were analyzed using the nonparametric Mann–Whitney tests or Welch's ANOVA.

To evaluate the effect of water, beta-alanine and jasmonic acid dipping treatments after one week of thrips infestation silver damage data were compared to non-dipped control cuttings using an unpaired Student's t-test. Likewise, differences among same treatment groups following an infestation period of two weeks were analyzed by a Student's t-test but, were square root transformed prior to analysis to meet normality assumptions. The test for normality, examining standardized skewness and the Shapiro-Wilks test, indicated that the data for mine and pupae counts were statistically non-normal. Therefore, differences in the total number of mines and total number of pupae between IBA-coated and non-coated control cuttings were analyzed by Mann–Whitney tests with a 5 % level of significance. An unpaired Student's t-tests was used to assess significant differences in number of leaves between both groups.

To investigate the effect of dipping treatments on leaf miner resistance, one-way ANOVA was used to analyze differences in total number of mines among groups. However, for pupae counts, the Levene's F test revealed that the homogeneity of variance assumption was not met ($p = 0.046$).

Therefore, a Welch's ANOVA followed by Games-Howell's post hoc was used to assess significant differences in total number of pupae among dipping treatments. Differences in emergence of pupae (emerged and non-emerged) were analyzed by a Welch's t-test. Data transformations were performed to significantly reduce heteroscedasticity and normalize residuals of PPO levels, but failed to meet the assumption of normality. Subsequently, reciprocally transformed PPO data were analyzed by Mann-Whitney test.

To study the underlying mechanisms of resistance (i.e. induction or priming), silver damage data between control and water dipped cuttings were analyzed using the Student's t-tests. Likewise, for the time-course experiment, differences in PPO levels between control and water dipped plants sprayed with MeJA or mock at each time point were tested using Student's t-tests whereas, PPO activities, before and after thrips infestation, were analyzed by Generalized Linear Models (GLM)) using linear distribution and identity link functions. Moreover, GLM were used to analyze the effect of dipping treatment, coating treatment and their interaction on silver damage symptoms, PPO activity, hormone concentrations and dry weight. Differences among groups were tested by Fisher's LSD post-hoc test. Differences in all analyses were considered significant at $p < 0.05$. As an indication which plant hormone variables would best predict variation in silver damage among treatments, multiple backward linear regression analysis was performed according to the Akaike information criterion (AIC). Variables were removed from the full model when the variance explained did not significantly improve the model (α=0.05). Among the five generated models, the most significant model, retaining a set of two strong predictors of silver damage, were further analyzed using GLM. For comparison of dry-powder application and liquid dips with IBA, square root transformed silver damage data were analyzed by Kruskal-Wallis followed by Dunn's test with Bonferroni correction for multiple comparisons. All statistical analyses were conducted with SPSS v. 24 software (IBM; SPSS Inc., Chicago, IL, United States).

Results

Effect of bio-insecticidal pre-pretreatments on thrips resistance

In preliminary experiments we observed that chrysanthemum cuttings were more resistant to thrips, i.e. displayed less silver damage, than untreated cuttings when the basal portion of cuttings were dipped in a solution with β-alanine (Supplementary figure S1; ($t(7) = 2.149$, $p = 0.069$). Therefore, we further investigated the potential of basal liquid dipping treatments, using aqueous solutions of β-alanine, as a possible strategy to enhance thrips resistance of chrysanthemum cuttings during their early vegetative stage. In comparison to non-dipped control cuttings, water dipping, but not β-alanine significantly reduced silver damage when cuttings were infested with thrips for one week (Figure 1A; $t(23) = 2.137$, $p = 0.043$). Furthermore, upon dipping in jasmonic acid, thrips-associated feeding damage was markedly reduced by 65% relative to the control ($t(18) = 4.680$, $p < 0.001$). Following an infestation period of two weeks, the effect of β-alanine dipping treatment was highly significant (Figure 1B; $t(46) = 3.623$, $p = 0.001$) however, did not differ significantly from water dipping

with respect to reduction in silver damage (Figure 1B). JA dip reduced silver damage by two-fold as compared to the control ($t(28) = 5.439$, $p < 0.001$). Taken together, these observations indicate that water dipping of IBA-coated cuttings is involved in enhancing resistance against thrips. Moreover, dipping treatments had no negative effect the total dry mass of cuttings ($F(7, 79) = 1.31$, $p = 0.257$).

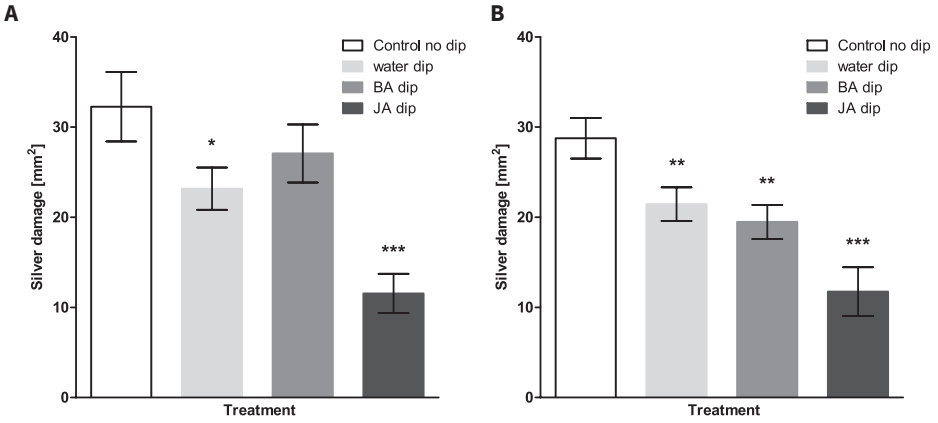

Figure 1. Effect of basal liquid dips on chrysanthemum resistance against western flower thrips (WFT). The basal portion of stem cuttings were dipped in 100 mg/ml of β-alanine (BA), water or 5 mM JA. Untreated, non-dipped, cuttings served as control. All commercially provided cuttings were pre-coated with indole-3-butyric acid IBA (Chryzotek beige 0.4 %). Two weeks post treatment, cuttings were infested with 20 adult thrips for a period of **(A)** 7 days or **(B)** 14 days. Silver damage symptoms were visually scored and expressed as damaged leaf area in mm². Means for water dipping and β-alanine represent pooled cumulative silver damage of three dipping timepoints (30, 45 and 60 minutes). Data are presented as mean ± SEM. Asterisks indicate significant differences in comparison to non-dipped control as determined by an unpaired Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Water dipping of IBA-coated cuttings confers protection against thrips

The observations of reduced silver damage upon water dipping gave rise to the hypothesis that auxins may exert an antagonistic effect on the jasmonic acid signaling pathway, and consequently, increase WFT susceptibility of cuttings commercially pre-coated with auxins. In order to evaluate the influence of auxin on thrips susceptibility, unrooted cuttings with and without IBA were infested with adult thrips after a rooting period of two weeks. Furthermore, we evaluated the effect of water dipping at three different time points namely; 0 – 30 – 45 and 60 minutes (Figure 2). Significant differences among treatments were observed, both for dipping time and auxin coating (GLM: Wald $\chi^2 = 9.92$, $p = 0.019$ and Wald $\chi^2 = 4.55$, $p = 0.033$, respectively). The time by coating interaction was not significant (GLM: Wald $\chi^2 = 3.02$, $p = 0.388$). Auxin coating of cuttings significantly affected silver damage symptoms however, mean comparisons within dipping time revealed there were no significant differences between IBA-coated and non-coated cuttings under control conditions (i.e. non-dipped cuttings at t=0). Additionally, at 45 and 60 minutes no significant differences were observed between cuttings pre-treated with IBA and untreated cuttings. Interestingly, however, water dipping of IBA treated cuttings significantly increased thrips resistance. The response to water dipping was independent of the time. After 30 and 60 minutes plants displayed significantly less silver damage.

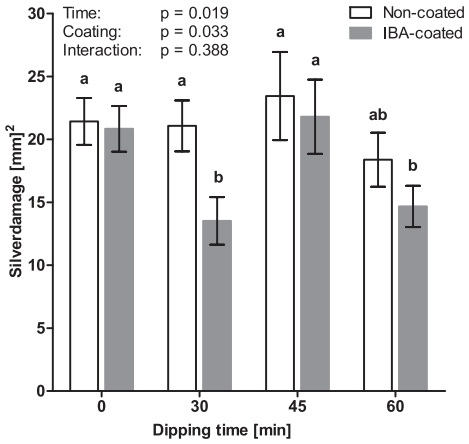

Figure 2. Effect of IBA coating and water dipping on chrysanthemum susceptibility against thrips. Basal cut ends (cv. Baltica), in the presence or absence of the powder formulated rooting hormone IBA, were dipped in water at various durations. Two weeks post treatment, cuttings were infested with 20 adult thrips for a period of one week. Cumulative silver damage data are presented as mean ± SEM (n=10). Different letters denote significant differences among groups as determined by GLM followed by Fisher's LSD test ($P < 0.05$). The overall effects of dipping time, auxin coating and their interaction are indicated in the graph.

Silver damage symptoms were reduced up to 35 % compared to plants grown from non-dipped IBA-coated cuttings. In contrast, in the absence of exogenous auxin coating, cuttings displayed no significant differences following water dipping.

Water dipping of IBA-treated cuttings reduced pupae emergence

The unexpected benefit of water dipping triggered our curiosity to further evaluate whether such an approach could improve resistance against other herbivores. Therefore, we evaluated the effect of water dipping treatments on celery leaf miner resistance, a second important pest insect species of chrysanthemum. Two separate experiments were performed to explore the role of IBA in leaf miner resistance. Firstly, we investigated the physical effect of the rooting hormone IBA on leaf miner resistance by comparing IBA-coated and non-coated cuttings (Supplementary Figure 2). We observed that the mere physical presence of IBA does not influence susceptibility of chrysanthemums to leaf miners. Total number of mines ($U = 74.5$, $p = 0.114$) as well as the number of pupae ($U = 91$, $p = 0.372$) were not affected by exogenous application of IBA to cut ends (Supplementary figs. 2A and 2B, respectively). The growth of cuttings, on the contrary, was significantly influenced by IBA. After two weeks of rooting, the number of leaves in cuttings pre-treated with the rooting hormone IBA (9.60 ± 0.25) was significantly higher than the untreated control group (8.72 ± 0.23) ($t(28) = -2.536$, $p = 0.017$). Additionally, in a second experiment we further assessed the effect of water dipping in the presence of the rooting hormone IBA on leaf miner (Figure 3). Group means of the total number of mines did not differ significantly ($F(4, 59) = 1.08$, $p = 0.374$). In contrast, the number of pupae was significantly affected by dipping ($F(4, 28.52) = 10.59$, $p < 0.001$). The amount of time cuttings were dipped in water had no effect on pupation. Post hoc comparisons revealed that non-dipped control cuttings and

water dipped cuttings were similar to each other, but were significantly different from the jasmonic acid treated group (Figure 3B). It is noteworthy, however, that the effect of water dipping becomes more evident when the ratio between emerged and non-emerged pupae is examined. Leaf miner larvae pupate in hibernacula within mines and emerge before or after leaf abscission. Interestingly, upon further evaluation a significantly lower rate of emergence was observed for groups receiving a dipping treatment whereas, in comparison to the non-dipped control group, no differences for non-emerged pupae were observed (Figure 4A). The number of pupae successfully emerging from chrysanthemum leaves was significantly lower after 30 and 45 minutes of dipping ($t(15,27) = 2.55$, $p = 0.022$ and $t(16,69) = 2.18$, $p = 0.044$, respectively). For cuttings that were dipped in water for 60 minutes we also observed a reduction although not statistically significant ($t(20,64) = 1.65$, $p = 0.115$). Treatment of cut ends with JA significantly reduced emergence rate by 97% as compared to non-dipped control cuttings ($t(12,08) = 3.93$, $p = 0.002$).

To investigate whether reduction in pupae emergence rates were related to induction of defenses, we assayed the activity of the defense-related protein polyphenol oxidase (PPO) by sampling leaf material prior to infestation (Figure 4B). An irregular pattern of PPO activity was observed in water and JA treatment groups. Activities of PPO were not significantly stimulated following dipping treatments suggesting that enhanced resistances were not explained by direct induction. Nonetheless, upon a dipping duration of 30 minutes in water, PPO levels were only marginally statistically enhanced in comparison to the non-dipped control ($U = 19.0$, $p = 0.068$).

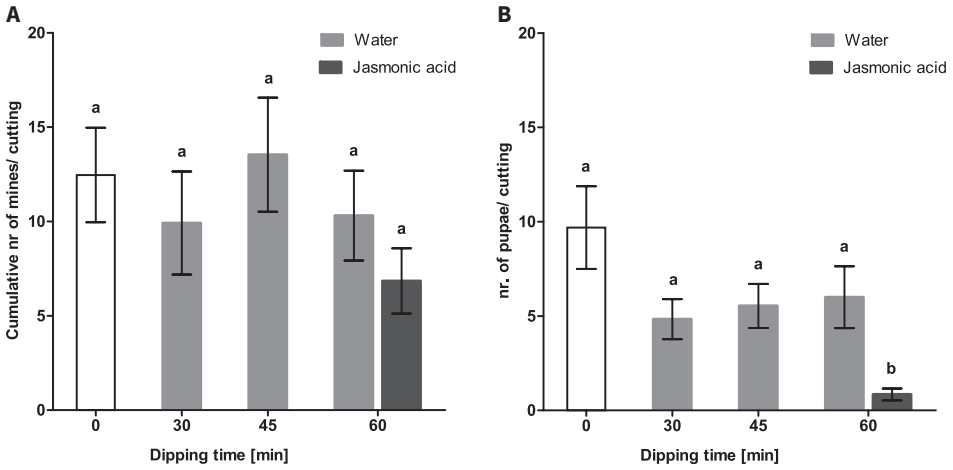

Figure 3 Effect of water dipping and jasmonic acid on leaf miner resistance (*Liriomyza trifolii*). Unrooted cuttings, pre-coated with indole-3-butyric acid (IBA), were dipped in water for various durations or in a solution of 5 mM jasmonic acid for 60 min. Two weeks post treatment, cuttings were individually caged and infested with four flies for 24 hours. **(A)** Total number of mines were scored after 3 days, whereas the number of pupae were counted after one week **(B)**. Data are represented as mean ± SEM, n=13. Different letters indicate significant differences among treatments as determined by **(A)** Fisher's least significant difference (LSD) test or **(B)** Games–Howell at $p < 0.05$.

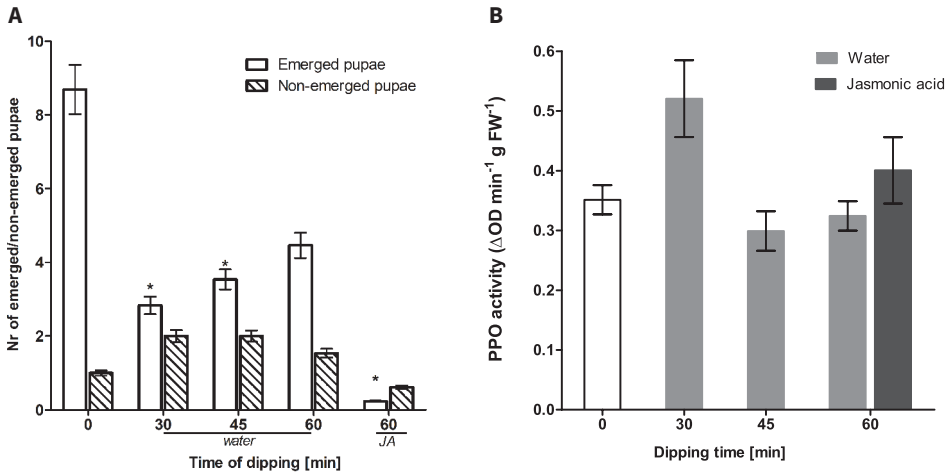

Figure 4. Effect of dipping on the leaf miner pupation and induction of polyphenol oxidase (PPO) activity. Cut ends of chrysanthemum, pre-treated with 0.4% IBA, were dipped in water for various durations and in a solution of 5 mM jasmonic acid for 60 min. Non-dipped cuttings, indicated at t=0 in the graph, serve as control. **(A)** Number of pupae emerging from chrysanthemum leaves and non-emerged pupae were scored after one week (n=13). Asterisks indicate statistical significance in comparison to non-dipped control cuttings determined by Welch's t–test (*p <0.05). **(B)** PPO (n=8-10) was measured in the third leaf from the bottom prior to leaf miner infestation. Reciprocally transformed PPO data were analyzed by Mann-Whitney test. Data shown are representative of means ± SEM.

Water dipping does not induce nor prime for enhanced defenses

After evaluating the effect of auxin and dipping treatments on resistance to thrips and leaf miner, the most effective treatment was selected to further study the dynamics of induced resistance. We observed that efficacy of water dipping was most evident at 30 minutes. To demonstrate whether defense responses were induced directly or primed for potentiated expression upon thrips infestation, the activity of PPO was assayed by sampling leaf material before and after thrips infestation, respectively. Firstly, we investigated how water dipping of IBA-coated cuttings affected the induction of the JA-associated marker PPO and, concomitantly, the effect on resistance against WFT (Figure 5). In contrast to our previous observations, however, we found only a minor reduction in silver damage by water dipping and this was not significant ($t(38) = 1.45$, $p = 0.156$). The lack of enhanced resistance corresponds with the measured PPO levels. PPO activities were not stimulated upon water dipping treatment of cuttings (GLM, Wald $\chi^2 = 1.05$, $p = 0.307$). To further address whether water dipping primed plant defense, we assessed the induction of PPO activity under thrips infestation in control and water-dipped cuttings. Thrips infestation had no effect on the level of PPO (GLM, Wald $\chi^2 = 0.09$, $p = 0.763$).

Plants respond to exogenous methyl jasmonate (MeJA) with a myriad of inducible defense responses, including the production of anti-digestive proteins such as PPO (Constabel and Ryan, 1998). The similarities to defense responses triggered by herbivores allows to closely mimic wound-induced responses by artificial elicitation and hence, provides potential means to explore how dipping treatments of chrysanthemum cuttings contribute to enhanced thrips resistance. Therefore, in a

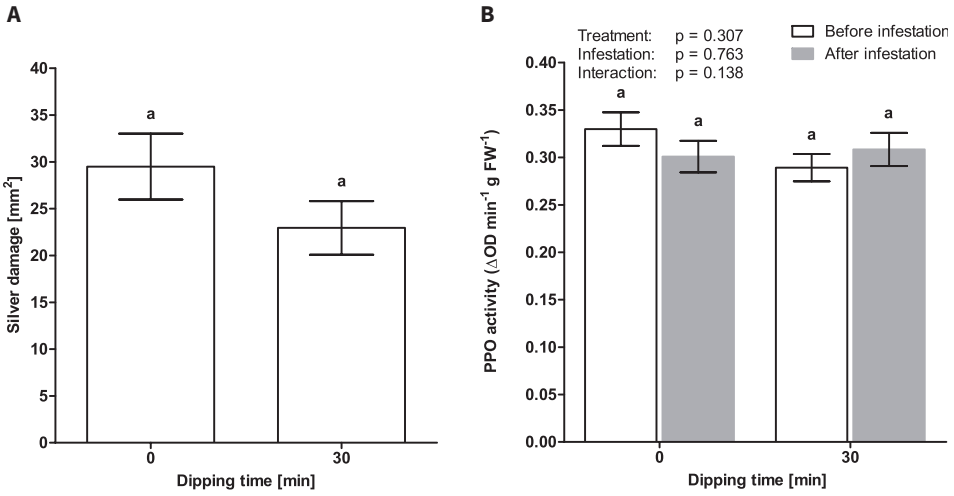

Figure 5. Effect of water dipping treatment on thrips-associated silver damage **(A)** and polyphenol oxidase (PPO) activity **(B)**. Cut ends of chrysanthemum cultivar Baltica, pre-coated with the powder formulated rooting hormone IBA (Chryzotek beige 0.4%), were dipped in water for 30 min. Two weeks post treatment, cuttings were infested with 20 adult thrips or sampled for PPO measurements before infestation. One week after thrips infestation, silver damage symptoms were visually scored and expressed as damaged leaf area in mm^2 and subsequently sampled for PPO activity after infestation. Data in the graphs represent mean ± SEM of 20 individual cuttings. The main effects of dipping time, infestation and their interaction are indicated in the graph.

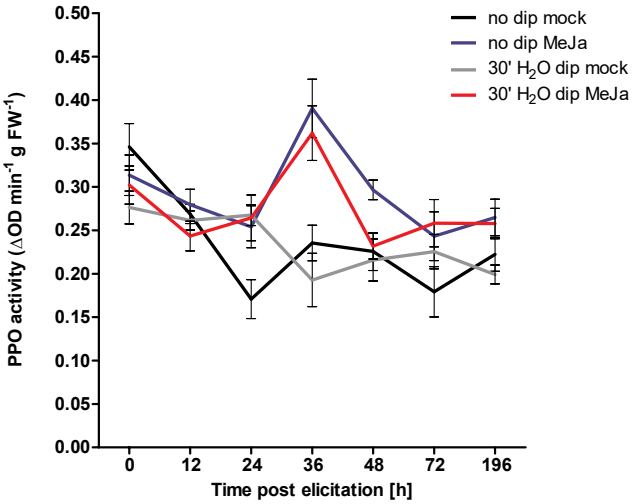

Figure 6. Effect of water dipping treatment on polyphenol oxidase activity (PPO). Comparative graph of PPO activity on a time course after MeJA elicitiation in water dipped cuttings and non-dipped control cuttings. Basal ends of chrysanthemum cuttings (cv. Baltica), coated with the powder formulated rooting hormone IBA (Chryzotek beige 0.4%), were dipped in water at for 30 minutes or left untreated. Two weeks after rooting, cuttings were directly harvested for PPO analysis (t = 0 min) or foliarly sprayed with 7,5 mM MeJA to mimick thrips infestation. Mock treated plants served as control. PPO activity was measured in the third leaf from the bottom. Values represent the mean of 10 replicates with SEM. Asterisks indicate significant difference at α=0.05 as determined by an unpaired Student's t-test.

further attempt to elucidate the role of auxin and water dipping treatments in establishing enhanced herbivore resistance, a time-course experiment was performed using MeJA for artificial induction of JA-associated defenses (Figure 6). In mock-treated plants PPO activities initially decreased and were then stabilized over time. PPO activities reached a peak level 36 h after methyl jasmonate (MeJA) application and then gradually declined at the end of the experiment. Exogenously applied MeJA significantly enhanced PPO activities by two-fold in water-dipped plants as compared to their corresponding mock-treated control (30 min H_2O dip-mock; t (18)=3.85, p = 0.001). Interestingly, the rate of PPO synthesis changed almost coordinately with non- water dipped plants and a similar trend was observed upon hormone treatment. PPO activity was directly responsive to exogenous MeJA treatment. Likewise, 36 h hours post elicitation, PPO levels were significantly amplified relative to the corresponding mock-control (t(18)=3.95, p < 0.001).

Standardization of IBA applied powder

Dipping treatments of chrysanthemum cuttings generally suffer from inconsistency. Many reasons can account for this situation among which a lack of standardized IBA formulation can be hypothesized. Indeed, within each batch of commercially provided unrooted cuttings, we observed considerable variation in the amount of IBA applied rooting powder at the basal cut ends (Supplementary Figure S3). In view of this, the current experiment aimed to reduce response variations by regulating factors that may potentially influence absorption of IBA through the cut base. To this end, the loading amount of powder applied IBA at the cut base and duration of water dipping were systematically studied (Figure 7A). Silver damage did not significantly differ among various dipping treatment (GLM: Wald χ^2 =1.41, p = 0.236). Similarly, exogenous powder application neither affected silver damage symptoms (GLM: Wald χ^2 = 6.73, p = 0.081). However, a significant interaction was observed between auxin treatment and dipping time (GLM: Wald χ^2 = 13.91, p = 0.003). The least amount of silver damage was observed in non-coated cuttings under control conditions (i.e. control cuttings at t= 0 min). In the absence of a water dipping treatment, powder formulated hormonal application at the basal cut ends, irrespective of the type and concentration, significantly increased silver damage whereas, with increased duration of water dipping these negative effects were not observed.

These results contrast our previous observations, where no significant differences in silver damage were observed between IBA-coated and non-coated control cuttings (Figure 2). Upon water dipping, silver damage was significantly increased in control cuttings free of powder applied hormones while an opposite trend was observed for powder-coated cuttings. Notably, upon water dipping, IBA-coated cuttings, at a concentration of 0.4%, displayed considerably lower silver damage symptoms in comparison to non-dipped IBA cuttings. Silver damage symptoms were significantly reduced by approximately 33%. By contrast, the beneficial effect of water dipping was far less evident for cuttings treated with 0.8% IBA where, water dipping only marginally reduced silver damage at 45 minutes. Furthermore, powder coating, but not water dipping, significantly influenced dry mass of chrysanthemum cuttings (GLM: Wald χ^2 = 52.81, p < 0.001 for coating; Wald χ^2 = 0.40, p = 0.527 for powder coating and Wald χ^2 = 2.16, p = 0.539 for the interaction). Among hormone concentrations, application of 0.8% IBA to the cut base of the stem adversely affected dry mass (Figure 7B).

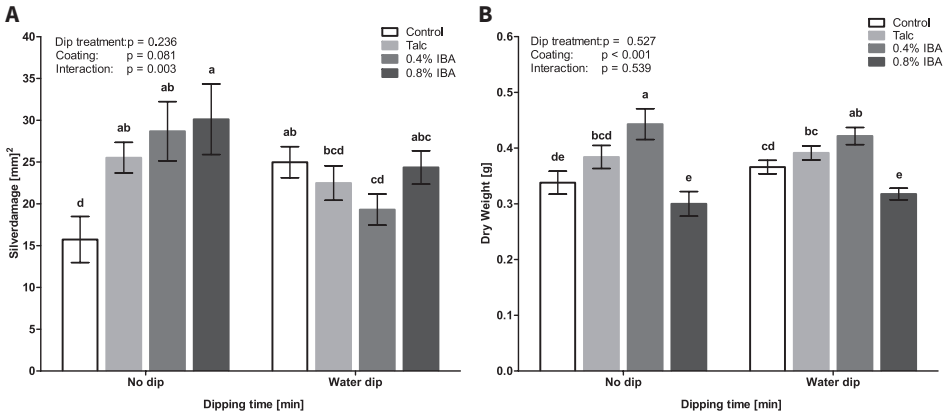

Figure 7. Effect of auxin formulation and duration of dipping on **(A)** thrips-associated feeding damage and **(B)** dry mass. Standardized powder treatments include pre-wetting of cutting base in water prior to coating in talc or 0.4 and 0.8% indole-3 butyric acid (IBA). Chrysanthemum cuttings were individually placed in thrips proof cages and were exposed to adult thrips released at rate of 20 thrips per cutting. One week post infestation, silver damage symptoms were visually scored. Data are expressed as damaged leaf area in mm^2 (means ± SEM). Means for water dipping represent pooled cumulative silver damages of three different dipping durations. Different letters denote significant differences among groups as determined by GLM followed by Fisher's LSD test ($p < 0.05$). The overall effects of dipping treatment, coating and their interaction are indicated in each graph.

Hormonal profiling

In an attempt to further elucidate factors underlying the phenomenon of enhanced resistance upon water dipping of IBA-coated cuttings, six major plant hormones were measured two weeks after dipping treatment (i.e. before thrips infestation). Multiple backward regression analysis was used to reveal the relative importance of plant hormones by eliminating variables with a low level of significance (Supplementary Table S1 and S2). The most significant model revealed that jasmonic acid and its isoleucine conjugate, JA-ile, were significant predictors of silver damage among different treatments. The most significant model revealed that jasmonic acid and its isoleucine conjugate, JA-ile, were significant predictors of silver damage among different treatments ($F(2, 13) = 5.03$, p = 0.024, R2 = 0.436). Subsequently, generalized linear models were applied to assess the main effects of cutting treatment and dipping time, as well as their pairwise interaction on JA and JA-ile (Table 1). The concentration of jasmonic acid varied significantly among treatments (GLM: Wald $\chi^2 = 6.75$, $p = 0.080$ for dipping time; Wald $\chi^2 = 39.50$, $p < 0.001$ for cutting treatment and Wald $\chi^2 = 29.90$, $p < 0.001$ for the interaction). Jasmonic acid-isoleucine concentration, on the other hand, was only influenced by dipping time (GLM: Wald $\chi^2 = 9.45$, $p = 0.024$ for dipping time). Coating of cuttings with talc or IBA only marginally affected the concentration of JA-ile (Wald $\chi^2 = 7.21$, $p = 0.065$ for coating and Wald $\chi^2 = 13.02$, $p = 0.162$ for the interaction). However, reductions in silver damage were not accompanied by marked increases in jasmonic acid nor by upregulation of its isoleucine conjugate and thus, do not support a synergistic nor an antagonistic mode of action of IBA.

Table 1. Hormonal profiling upon cutting treatments. Hormone concentrations are expressed as mean in ng/mg freeze dried leaf material ± SEM (n=5).

	Cutting treatment	Dipping time	Hormone concentrations					
			Ja-Ile			JA		
Treatments	Control	0 min	0.11 ± 0.05	bcde		58.91 ± 8.61	cdef	
		30 min	0.05 ± 0.01	de		50.31 ± 7.07	fg	
		45 min	0.04 ± 0.01	de		84.88 ± 8.30	ab	
		60 min	0.09 ± 0.05	cde		77.28 ± 7.02	abd	
	Talc	0 min	0.33 ± 0.12	ab		82.55 ± 7.00	ab	
		30 min	0.04 ± 0.01	de		75.89 ± 5.26	abc	
		45 min	0.41 ± 0.26	a		92.40 ± 10.12	a	
		60 min	0.06 ± 0.04	cde		75.43 ± 10.15	abc	
	IBA 0.4%	0 min	0.08 ± 0.03	cde		69.83 ± 4.80	bcd	
		30 min	0.21 ± 0.16	abcde		68.74 ± 11.59	bcde	
		45 min	0.20 ± 0.03	abcde		61.11 ± 6.46	cdef	
		60 min	0.02 ± 0.01	e		40.33 ± 5.27	g	
	IBA 0.8%	0 min	0.26 ± 0.09	abcd		53.70 ± 5.79	defg	
		30 min	0.12 ± 0.06	bcde		48.14 ± 5.41	fg	
		45 min	0.29 ± 0.11	abc		51.32 ± 3.40	efg	
		60 min	0.14 ± 0.05	bcde		61.42 ± 7.34	cdef	
GLM	Coating	Wald χ^2	7.21	p	= 0.065	39.50	p	< 0.01
	Dipping time	Wald χ^2	9.48	p	= 0.024	6.75	p	= 0.08
	Interaction	Wald χ^2	13.02	p	= 0.162	29.90	p	< 0.01

Abbreviations: indole-3-butyric acid (IBA); jasmonic acid (JA); JA-isoleucine (JA-Ile). Different letters denote significant differences among treatment groups as determined by Generalized Linear Models (GLM) followed by LSD at $p < 0.05$.

Comparing dry-dip rooting powder with water-based rooting solution

Because of possible confounding effects exerted by talc, we aimed to differentiate between the impact of IBA and that of the carrier chemical talc contributing to the absence of plant defense responses. In this context, basal liquid dips were performed using concentrations equimolar to powder applied IBA. Concomitantly, commercially provided cuttings pre-coated with 0.4% IBA were included in the experiment to compare the relative effectiveness of application and possibly the influence of IBA absorption through the cut base. Figure 8 shows that, in general, non-coated cuttings displayed the least amount of silver damage whereas, strikingly, water dipping of IBA-coated cuttings yielded the highest level of silver damage. The Kruskal-Wallis test showed that there was a statistically significant difference in silver damage symptoms between the different treatments groups, H(4) = 17.138, $p = 0.002$. However, we observed no evidence that auxin supplementation enhanced reduced feeding symptoms. With the exception of water dipped IBA-coated cuttings, symptoms were equal across all groups. Under control conditions, when cuttings did not receive a water dipping, an increase in silver damage was observed in IBA-coated cuttings (32.3 ± 6.2), although this effect was not significantly different from non-coated cuttings (19.1 ± 3.4).

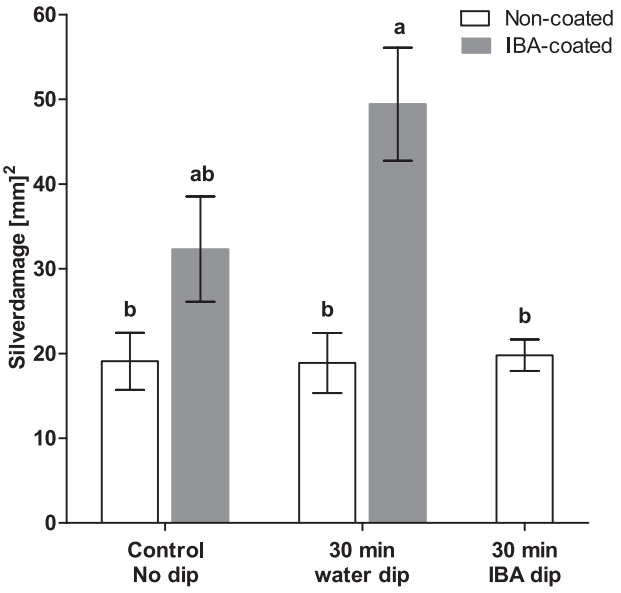

Figure 8. Effect of powder and liquid auxin formulations on silver damage. Unrooted indole-3-butyric acid (IBA)-coated (cv. Baltica) cuttings were dipped in water for 30 minutes. Non-coated cuttings were dipped in an equimolar concentration of 4000 ppm or water. Control cuttings did not receive a water dipping treatment. Bars (mean ± SEM, n=13 -15) represent the cumulative silver damage expressed as damaged leaf area in mm^2 Different letters indicate significant differences among groups as determined by Kruskall-Wallis followed by Games-Howell multiple post hoc comparison ($p < 0.05$).

Discussion

The exploitation of various secondary metabolites as defensive compounds holds great promise as a sustainable strategy for the control of pest insects (Cantrell et al., 2012; Lorsbach et al., 2019). Many secondary metabolites display interesting bioactivities, which either act directly as a result of their insecticidal properties or are mediated through the plant by induction of defenses. In the present study, we evaluated the effect of various basal liquid dipping treatments as a pre-propagation treatment to enhance resistance against thrips, early in the production cycle of chrysanthemums. The majority of experiments exploring the potential of bio-insecticidal dips are often carried out as immersion treatments. These studies mainly evaluate this approach as a postharvest disinfestation strategy of propagative cuttings in order to manage insect populations prior to shipment. Furthermore, such intervention strategies allow to control pest populations prior to entering the production cycle, and could potentially reduce reliance on chemical pesticides (Buitenhuis et al., 2006). In the present study, we explored naturally occurring plant compounds as a preventative means to enhance plant defenses against two of the most economically important pest insects of Chrysanthemum; WFT and leaf miner.

Among the defensive metabolites, β-alanine has frequently been implicated in resistance against herbivores of numerous taxa, including western flower thrips (Leiss et al., 2013). In preliminary

experiments we observed that chrysanthemum cuttings were more resistant to thrips, i.e. displayed less silver damage, than untreated cuttings when the basal portion of cut ends was dipped in an aqueous solution of β-alanine. Silver damage symptoms were reduced by 36% as compared to non-dipped cuttings. While the effect was comparable with that of Abamectin stem dipping, it should be noted that Abamectin acts as a contact insecticide and has a very limited systemic activity. Subsequently, in seeking to optimize the dipping treatments of chrysanthemum cuttings, we surprisingly observed that thrips associated feeding damage was remarkably lower in water dipped cuttings (Figure 1). The scientific practice of including an appropriate control had serendipitously yielded an unexpected benefit. Because the base commercial chrysanthemum cuttings is covered with rooting power containing auxin, this outcome raised two potential hypotheses on the role of auxins in relation to plant-insect defenses suggesting that either (1) antagony or (2) synergy could explain our observations. Firstly, it can be hypothesized that exogenous application of the growth regulating hormone, IBA, attenuated thrips resistance by exerting an antagonistic effect on the JA-signaling pathway. While jasmonates are generally recognized as the prominent hormone in plant defense against herbivores, recent studies revealed that auxins may also have a key role in modulating this process (Zhao, 2018). The assumption of a dual role of auxins is supported by several lines of evidence. A number of studies have demonstrated an inhibitory effect of exogenously applied auxins on JA-biosynthesis. A direct prediction of our first hypothesis is that the physical presence of IBA on stem cuttings promotes thrips susceptibility. We tested this hypothesis by performing water dipping treatments in the presence and absence of the rooting hormone IBA. Silver damage symptoms were significantly different among treatments and were influenced both by period of dipping and coating treatment of cuttings (Figure 2). When cuttings received no water dipping treatment, we observed no significant differences in silver damage between IBA-coated and uncoated cuttings. Likewise, we found no significant differences between coated and non-coated cuttings when they were dipped for 45 and 60 minutes. The present findings therefore, do not support the hypothesis that auxins play a direct role in promoting susceptibility of cuttings to thrips. Intriguingly, water dipping treatments in the presence of IBA-coating were shown to significantly reduce silver damage symptoms while in the absence of exogenous auxins, cuttings displayed no significant differences in thrips damage following water dipping. Consequently, removal of externally applied hormone powder by water dipping treatments could have mitigated the antagonistic effect of auxin. Alternatively, rather than removal of auxins, water dipping treatments could have enhanced the uptake of exogenously applied auxin, and subsequently leading to increased resistance through a synergistic mode of action with JA.

The beneficial effect of water dipping triggered our curiosity to further evaluate whether such an approach could improve resistance against multiple herbivores. As such, we evaluated the effect of dipping treatments on celery leaf miner resistance, a second important pest insect species of chrysanthemum. In agreement with our thrips data, it is clear that the mere physical presence of IBA did not explain susceptibility of chrysanthemum cuttings to *Lyriomyza trifolii*. Furthermore, we observed that water dipping had no direct effect on the number of mines (Figure 3A). Although seemingly lower, no significant differences were detected in total pupation (Figure 3B). However, the

number of pupae successfully emerging from leaves was significantly lower after water dipping (Figure 4A). The most prominent effect was observed in JA treated cuttings. Cut ends dipped in JA significantly reduced the emergence rate of pupae by 97%. In order to determine whether these enhancements were associated with JA-dependent defense traits, we measured the activities of polyphenol oxidase (PPO). Induced resistance may result from a direct activation of defense mechanisms, including increased basal levels of defense-related proteins such as PPO which serve an anti-nutritive role by reducing the digestibility of dietary protein (Felton, 2005; Conrath et al., 2006; Robert-Seilaniantz et al., 2011). Although significant reductions in the number of emerged pupae were observed, none of the dipping treatments stimulated PPO activity. Surprisingly, JA dipping did not induce PPO levels either. This could be explained by the time gap following dipping treatment and PPO measurements. Furthermore, rather than direct induction of defenses, it is plausible that dipping treatments of auxin-coated cuttings primed for enhanced defense reactions. The priming defense processes remain dormant until herbivore infestation and reflects a cost-effective approach by which the plant can avoid expending energy under low pest pressure (Conrath et al., 2006; Huot et al., 2014).

To establish whether enhanced resistance in chrysanthemum was associated with priming of the plant defensive capacity, a comparative study was undertaken. In the first experiment, we compared thrips-mediated induced responses in IBA-coated chrysanthemum cuttings following a 30 minute water dipping treatment. Again we found a reduction of silver damage after water dipping but this time this was not statistically significant. (Figure 5A). In parallel, as a marker of plant defense responses, the activity of PPO was measured two weeks post treatment (i.e. before thrips infestation) and one week post thrips infestation (Figure 5B). We observed no induction of PPO activities upon 30 minutes of water dipping. Furthermore, no significant differences were observed between thrips-infested and non-infested chrysanthemum cuttings . These observations suggest that neither induction nor priming of plant defenses are involved. However, the current experimental set-up as well as the minor effect on silver damage, do not allow us to draw a conclusive picture of underlying mechanisms and indicate the necessity for further study.

Activation of the JA signaling pathway through exogenous application of jasmonates, such as the volatile form of JA methyl jamonate (MEJA), is reported to induce the expression of the defensive protein PPO more efficiently than JA (Farmer and Ryan 1990; Jang et al., 2014; Jiang and Yan et al.,2018). MeJA-mediated induction of PPO was assayed as a means to mimic herbivore-induced responses (Constabel and Ryan, 1998) and hence, explore which role dipping treatments in the presence of auxin play in establishing resistance against thrips. Subsequently, a separate time-course experiment was performed to assess water dipping effects on artificially induced plant defense responses triggered by exogenous application of MeJA. The time course of PPO levels in cuttings treated with MeJA peaked after 36 hours for both the non-dipped and water dipped plants. Taken together, these experiments suggest that water dip treatments in the presence of IBA do not directly induce nor prime JA related defenses and, possibly operates independently from proteinaceous mediators such as PPO.

Besides their well-known defensive role, PPO is an important co-factor and acts as a stimulant of adventitious root formation by catalyzing the condensation between phenolic compounds and IAA

(Nag et al., 2001; Rout et al., 2006). Exogenous auxin treatment increases PPO activity while, decreasing the activity of indoleacetic acid oxidase (IAAO) (Zhang et al., 2017; Yan et al., 2017). peak values of PPO activity induced by exogenous auxin, appear to occur at different time points, depending on the plant species and hormone treatment. For example, in 1-naphthylacetic acid treated cuttings of hybrid aspen, PPO levels significantly increased 6 days after treatment (Yan et al., 2017) whereas, Zhang and co-workers (2017) reported a PPO peak value 45 days after planting IBA treated stem cuttings of *Malus hupehensis*. Future studies investigating the signaling pathways that govern the expression of defenses could contribute further to our understanding of mechanisms underpinning the processes of enhanced herbivore resistance. More specifically, aiming at understanding the early signaling events, by performing time course experiments during rhizogenesis may elucidate whether the defenses activated by water dipping are mediated through changes in PPO levels.

Considering earlier conflicting results concerning defense responses of chrysanthemum cuttings to water dipping, we emphasize the importance of standardizing hormone application. Within batch variations in hormone application may potentially have led to varying defense responses. Despite these extensive efforts, dipping and coating treatments continued to suffer from a high level of irreproducibility in thrips responses. Contrary to our earlier observations, we observed that Baltica cuttings under control conditions, i.e. 0 minutes of water dipping, exhibited significantly lower amounts of silver damage symptoms in the absence of talc-based IBA coating suggesting a potential antagonistic action of IBA. These contrasting results, as compared to our previous experiment, leads us to make two comments. The first concerns a plausible explanation of plant responses in relation to their water status. One of the main constraints in large-scale production of unrooted cuttings is the time delay between excision of cuttings from stock plants and insertion into a rooting environment. Once a stem is cut from its mother plant, its nutrient and water supply is lost. Traditionally, herbivorous insects are thought to exhibit enhanced performance on water-stressed host plants due to induced changes in plant physiology, largely through their effects on nitrogen availability (Huberty and Denno, 2004). One of the first physiological changes to occur in water deficit plants, is the loss of cell water content and turgor, which in turn influences plant resistance and susceptibility to herbivore attack. Whilst earlier we have demonstrated that, in the absence of exogenously applied IBA, water dipping of cut bases did not significantly affect silver damage symptoms ,the results obtained in the standardized experiment show a different trend. Surprisingly, water dipping of control cuttings free of powder applied hormones negatively affected silver damage and, symptoms were markedly increased by 37% relative to non-dipped cuttings(Figure 7). Our observations implicate that a positive cell turgor promotes susceptibility of chrysanthemum cuttings by enhancing thrips performance.

Notwithstanding, in line with our previous results, we observed that water dipping significantly reduced silver damage in cuttings treated with 0.4% IBA as compared to its corresponding non-dipped control (Figure 7). However, there was no clear consensus in the levels of auxin along with the associated improvement in silver damage symptoms. IBA application at basal cut ends did not increase endogenous levels of IAA in comparison to non-coated cuttings (Table 1).

Notably, IBA-coating of cut bases elevated the susceptibility of cuttings to thrips, as manifested by

significant increases in silver damage at t = 0 minutes (Figure 7). Although these results suggest that IBA exerts an antagonistic effect on the JA-signaling pathway, talc might act as a confounder. Talc, a clay mineral composed of hydrated magnesium silicate with the chemical formula $Mg_3H_2(SiO_3)_4$), is a functional carrier in many agricultural products and is extensively used as an inert chemical for active premixed ingredients. Talc is a strongly hydrophobic and an extremely platy mineral. As they are naturally water-repellent, talc particles can form a barrier when they envelop other particles and thus reduce the evaporation and uptake of water by preventing the formation of hydrate bridges, which is used to enable longer storage periods (IARC, 2010). Hence, the level of water stress in cuttings, as a result of talc-based coating, may have negative consequences for herbivores, especially for those that rely on high tissue water content and turgor (i.e. osmotic) pressure.

Multiple backward linear regression analysis revealed that differences in silver damage among chrysanthemum treatments could be explained in a model comprising JA and JA-isoleucine. However, multiple pairwise comparisons yielded no consistent pattern how dipping and coating treatments in relation to hormones were associated with reductions in thrips-associated feeding damage. As such, we have no firm evidence for a causal antagonistic or synergistic relationship in auxin-mediated responses. Importantly, these results should be interpreted in the context of spatial and temporal limitations. Hormonal concentrations were measured prior to thrips infestation and thus, two weeks post treatment, such measurements do not necessarily encompass hormonal changes induced by treatment of cuttings.

In an effort to identify and avoid possible confounding effects of talc-powder, basal liquid dips were performed using concentrations equimolar to powder applied IBA. We detected no evidence that IBA supplementation by liquid dips improved thrips resistance as silver damage, with the exception of water dipped IBA-cuttings, was equal across treatments). Strikingly, upon water dipping, IBA-coated cuttings displayed significantly more silver damage as compared to non-coated cuttings.

The large variation within and between experiments as well as the contrasting results leads to our second remark and concerns the status of the mother stock which is, perhaps, of greater importance than the water status of plant cuttings. An explanation of the apparent disparity of plant responses to thrips-associated feeding patterns may be the phenotypic heterogeneity of plant cuttings. We speculate that the physiological status of stock plants is key to explaining the discrepancy in the variable and inconsistent performances of thrips on generated cuttings. Maternal life history traits may affect the progeny ,i.e. generated stem cuttings, and thus, despite being clonal in nature, cuttings can display different response patterns when facing the same infestation conditions. First and foremost, cuttings originated from Ethiopia or Uganda, and as such, the considerable variation in results is likely to be affected by environmental conditions. Cuttings used for exploring the physical effect of IBA as illustrated in Figure 2, were obtained from a production site located in Ethiopia whereas, in the follow-up experiment exploring standardized IBA-loading, cuttings were obtained from Uganda (Figure 7). Furthermore, discrepancies in experimental results could be linked to collection time of cuttings. Experiments conducted with cuttings from Ethiopia were harvested from mother plants in week 49 (December 2017) whereas, in the latter experiment for IBA standardization,

pruned cuttings from mother plants grown in a greenhouse located in Uganda differed by one week in harvest date (week 27 and week 28; July 2018). In addition to regional or climatic influences, it is important to note that growth conditions among these commercial greenhouses are optimized for each production site and, as such, may for example affect the nutritional status of stock plants (S. Kos, pers. comm.). This lends additional support to the contention that phenotypic heterogeneity involves genotype by environment interactions. Other hypothetical possibilities that influence physiological responses include; but are not limited to, substrate, cutting position along the canopy structure of the stock plant, age and the health of mother plants. Moreover, despite the fact that greenhouses provide a controlled and constant environment suitable for year-round production of chrysanthemum cuttings, variation in pest activities still exists from one season to the next (de Kogel et al., 1997; Hewitt et al., 2014).

Concluding remarks

In summary, water dipping of IBA-coated cuttings repeatedly reduced herbivory, both by thrips as well as by leaf miner. However, results were highly variable and inconsistent. Furthermore, separation of IBA and talc implies a possible confounding effect of the carrier chemical. Assessment of polyphenol oxidase activity indicates that neither direct induction nor priming of JA related plant defenses are involved. Although inroads have been laid, future experiments aiming at understanding the early signaling events, including hormonal signaling networks, from a more holistic perspective may help to explain the physiological basis involved in conferring protection against herbivores.
A possible explanation of the anomalies appears to hinge on the large phenotypical variation in cuttings generated from stock plants and warrants the necessity to improve intra- and inter- variability and reproducibility. Nonetheless, our study provides an interesting starting point to investigate alternative roles of auxins in commercial propagation and may add, in addition to their growth regulating function, a promising defense strategy to the horticultural toolbox of chrysanthemum propagators. In view of these findings, we advise that the putative defensive role of auxins should be exploited and integrated in coherent models of response and function in model plant species prior to translation to commercially important horticultural crops. Future work with auxin formulations is needed to determine whether such an approach is of commercial value over a broader range of herbivores.

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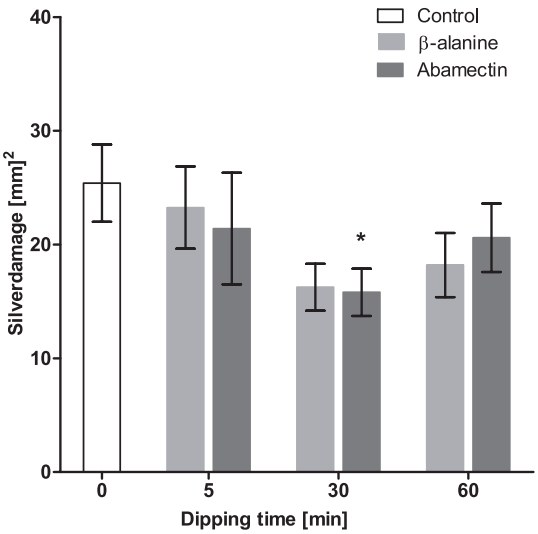
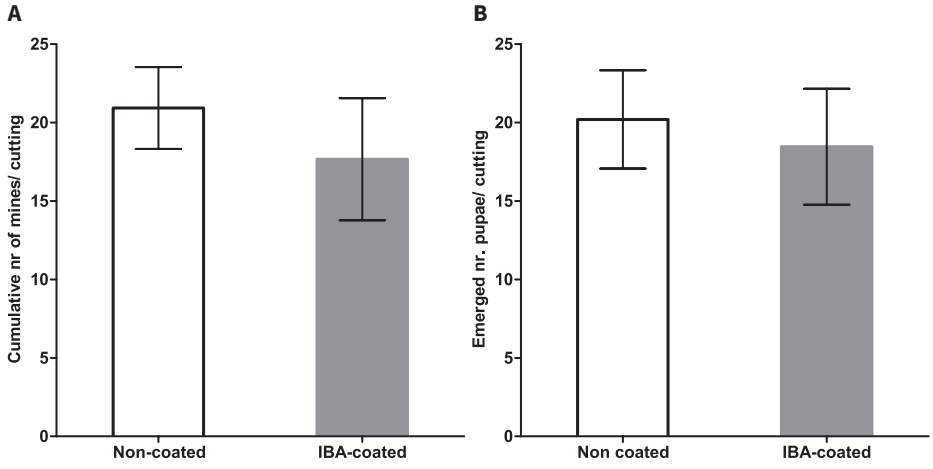
Supplementary Figures



Supplementary Figure S1. Efficacy of bio-insecticidal dips on chrysanthemum resistance against western flower thrips (WFT). The basal cut ends were dipped in 100 mg/ml of β-alanine or 0.3 ml/L abamectin (Avis EC, Syngenta, Syngenta Crop protection Inc., Greensboro, NC, USA) for various time points. Untreated, non-dipped cuttings served as control. Two weeks post treatment, cuttings were infested with 20 adult thrips. One week after thrips infestation, silver damage symptoms were visually scored and expressed as damaged leaf area in mm^2. Data represent cumulative silver damage and are presented as mean ± SEM of 5 replicates per treatment. Asterisks indicate significant difference at α=0.05 as determined by an unpaired Student's t-test.



Supplementary Figure S2. Effect of exogenously applied indole-3-butyric acid (IBA) on celery leaf miner (*Liriomyza trifolii*) resistance. Basal ends of chrysanthemum cuttings (cv. Baltica) were pre-coated with powder formulated rooting hormone (Chryzotek beige 0.4% IBA). Non-coated cuttings served as control. After 14 days of rooting, cuttings were infested with four one-day old leafminers (2 males and 2 females) for 24 hours. **(A)** cumulative number of individual mines and **(B)** the number of emerged pupae. Data represent means ± SEM of 15 replicates. Differences between IBA-coated cuttings and control plants were evaluated by the Mann-Whitney U test (two-tailed) at $p < 0.05$.

Supplementary Figure S3. Substantial variation in the amount of hormone applied rooting powder within a batch of 50 commercially provided Baltica cuttings. Cuttings at the left upper part of the image contain considerably more rooting powder than the lower part whereas, cuttings on the left upper part are loaded with a mediocre amount. Approximately 1 cm of the basal cut end is pre-coated with the rooting powder Chryzoteck beige 0.4% (Rhizopon, Hazerswoude-Rijndijk, the Netherlands) consisting of 0.4% indole-3 butyric acid (IBA) in talc. Baltica cuttings require IBA applied rooting hormones for quick root initiation.

Supplementary Table S1. Backward multiple regression models of independent predictors of variation in silver damage.

Model	Independent variables	B	SE	β	t	*p*
1	OPDA	0.049	0.055	0.212	0.891	0.396
	JA-ile	-22.390	10.812	-0.561	-2.071	0.068
	JA	0.183	0.094	0.583	1.951	0.083
	IAA	-0.054	0.111	-0.149	-0.484	0.640
	ABA	0.163	0.137	0.301	1.190	0.265
	SA	0.065	0.090	0.184	0.720	0.490
2	OPDA	0.045	0.052	0.194	0.860	0.410
	JA-ile	-23.247	10.249	-0.583	-2.268	0.047
	JA	0.158	0.075	0.504	2.099	0.062
	ABA	0.143	0.126	0.264	1.140	0.281
	SA	0.066	0.087	0.186	0.760	0.465
3	OPDA	0.048	0.051	0.210	0.955	0.360
	JA-ile	-19.522	8.827	-0.489	-2.212	0.049
	JA	0.146	0.072	0.465	2.021	0.068
	ABA	0.146	0.123	0.269	1.184	0.261
4	JA-ile	-19.950	8.783	-0.500	-2.271	0.042
	JA	0.159	0.070	0.507	2.253	0.044
	ABA	0.113	0.118	0.208	0.956	0.358
5	JA-ile	-21.308	8.639	-0.534	-2.466	0.028
	JA	0.176	0.068	0.562	2.594	0.022

Note: Independent variables: concentration of 12-oxo-phytodienoic acid (OPDA), jasmonic acid-isoleucine (JA-Ile), jasmonic acid (JA), indole-3-acetic acid (IAA), abscisic acid (ABA) and salicylic acid (SA). Abbreviations: B: unstandardized regression coefficient, β: standardized regression coefficient, SE: standard error Linear regression through the origin at $p < 0.05$, t-value and 2-tailed p-value.

Supplementary Table S2. Model summary of the backward elimination multiple regression analysis.

Model	Adj R^2	df 1	df 2	F	Sig.
1	0.257	6	9	1.865	.192[b]
2	0.314	5	10	2.374	.115[c]
3	0.340	4	11	2.935	.071[d]
4	0.345	3	12	3.636	.045[e]
5	0.350	2	13	5.030	.024[f]

Note: Abbreviations: Adj, adjusted; df, degrees of freedom. *p < 0.01 (two-tailed).
[a] Dependent variable: silver damage
[b] Predictors: (Constant), SA, IAA, OPDA, ABA, JAile, JA
[c] Predictors: (Constant), SA, OPDA, ABA, JAile, JA
[d] Predictors: (Constant), OPDA, ABA, JAile, JA
[e] Predictors: (Constant), ABA, JAile, JA
[f] Predictors: (Constant), JAile, JA